

Sedimentation properties of the scrapie agent

(subcellular fractionation/differential centrifugation/viral agent titration)

STANLEY B. PRUSINER*, WILLIAM J. HADLOW†, CARL M. EKLUND†, AND RICHARD E. RACE†

* Departments of Neurology and Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143; and † Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840

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ABSTRACT The sedimentation behavior of the scrapie agent in homogenates of spleen from infected mice has been determined. Approximately 90% of the scrapie agent was sedimented at an $\omega^2 t$ value of 3×10^{10} rad²/sec in a fixed-angle rotor. Sedimentation of the agent was not substantially affected by sonication or by treatment with the detergent sodium deoxycholate. The sedimentation profiles of the scrapie agent were similar to those observed for free polyribosomes, but differed from those exhibited by five other subcellular markers. Comparative studies showed that the sedimentation profiles of subcellular markers in spleen suspensions from mice infected with scrapie did not differ from uninoculated controls. These studies suggest that the scrapie agent is a discrete infectious particle which should be separable from cellular membranes.

To date, the scrapie agent has defied isolation and identification. Studies on the transmission and neuropathology of scrapie suggest that it is a reasonable prototype for the other subacute spongiform encephalopathies, kuru, Creutzfeldt-Jakob disease, and transmissible mink encephalopathy (1-4). The unusual physicochemical properties of the scrapie agent, its slow mode of replication, and its lack of detection by host defense mechanisms suggest that this causative agent is a novel infectious entity (5, 6). Unlike conventional viruses, the agent has not been seen by electron microscopy and has not been shown to be antigenic.

The unique resistance of the scrapie agent to inactivation by heat, formalin, and ultraviolet radiation, and its extremely small ionizing radiation target size have led several investigators to speculate that the agent may not contain a nucleic acid, but may be composed only of carbohydrate, or possibly protein (5, 6). To date, attempts to purify the scrapie agent have been generally unsuccessful and a hypothesis that the agent is an integral part of cellular membranous structures has evolved (2, 6). This "membrane hypothesis" also suggests that separation of the scrapie agent from cellular membranes may not be possible.

In part, the difficulties encountered by investigators attempting to purify the scrapie agent have been due to the inconvenient titration assay in mice. These titrations require care and observation of the mice over the 10- to 12-month period during which the disease develops (7). In order to develop a preparatory procedure for purification of the scrapie agent, we have studied the sedimentation characteristics of the agent in fixed angle rotors using the technique of analytical differential centrifugation (8). These studies indicate that the scrapie agent is probably a discrete infectious particle which can be sedimented at an $\omega^2 t$ value of 3×10^{10} rad²/sec. The sedimentation behavior of the agent was not substantially altered by sonication or by treatment with the anionic detergent sodium deoxycholate (DOC), and the agent has been found to sediment with

polyribosomes. The data suggest that it will be possible to isolate the scrapie agent.

METHODS

The comparatively rapid replication of the scrapie agent in spleen versus brain (9, 10) and the ease of fractionating spleen compared with brain suggested that our initial sedimentation studies might be more readily performed with spleen.

Female Swiss mice, 1-month-old, purchased from Charles River Laboratories, were inoculated intracerebrally with 0.03 ml of the mouse-adapted Chandler scrapie agent [10^6 mean infective dose (ID₅₀) units] (9, 11). The mice were sacrificed 42 days after inoculation. Spleens from 47 mice were minced in 20 mM Tris-HCl, pH 7.6/250 mM sucrose. A Potter-Elvehjem homogenizer was used to prepare a 20% homogenate (wt/vol). All procedures were performed at 4° unless otherwise noted. The homogenate was centrifuged in a Sorvall RC2B centrifuge at $121 \times g$ for 10 min and the supernatant fluid was removed. The pellet was rehomogenized in additional buffer and centrifuged again. After the pellet was washed and a third centrifugation was performed, the three portions of supernatant fluid were combined; the final suspension was 10% (wt/vol). The $121 \times g$ combined supernatant fluid was used as the starting material for all of the analytical differential centrifugation studies reported here.

Two-milliliter aliquots of the $121 \times g$ combined supernatant fluid were centrifuged in a Beckman 50 Ti fixed-angle rotor at specified speeds for given periods in cellulose nitrate tubes (8 mm diameter \times 50 mm length). In the experiments described below, the quantities of subcellular markers and scrapie agent in the supernatant fluid were plotted as a function of $\omega^2 t$, in which ω is the angular velocity of the centrifuge rotor in rad/sec and t is the time of centrifugation in sec.

The following enzymatic and chemical markers were used to identify subcellular organelles (12): succinate dehydrogenase (EC 1.3.99.1, SDH) (13); lactate dehydrogenase (EC 1.1.1.27, LDH) (14); alkaline (EC 3.1.3.1) and acid phosphatases (EC 3.1.3.2) (15); 5'-nucleotidase (EC 3.1.3.5) and glucose-6-phosphatase (EC 3.1.3.9) (16-18); cytochrome *c* reductase (EC 1.6.2.4) (19). RNA and DNA were measured by the procedure of Schneider, using orcinol and diphenylamine reagents, respectively (20). Commercial preparations of yeast RNA and calf thymus DNA were extracted with phenol and used as standards assuming an $A_{1\text{cm}}^{1\%} = 250$ for RNA and an $A_{1\text{cm}}^{1\%} = 200$ for DNA. Protein was determined by the method of Lowry *et al.*; bovine serum albumin was used as a standard (21).

Female Swiss mice of Rocky Mountain Laboratory stock were used to titrate the amount of scrapie agent in the supernatant fractions. Serial 10-fold dilutions of the fractions were

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Abbreviations: DOC, sodium deoxycholate; SDH, succinate dehydrogenase; LDH, lactate dehydrogenase; ID₅₀, mean infective dose.

Table 1. Distribution of subcellular markers in fractions of scrapie-infected spleen homogenates

Parameter	Fraction		
	Initial homogenate	121 × g supernatant	121 × g pellet
Protein (mg)	1017.0	547.0	432.0
RNA (mg)	64.6	46.2	32.3
DNA (mg)	380.0	16.6	386.0
Lactate dehydrogenase*	136.0	75.0	38.0
Alkaline phosphatase*	3.2	1.4	0.08
Glucose-6-phosphatase*	1.2	0.3	0.7
5'-Nucleotidase*	6.1	2.9	0
Acid phosphatase*	43.0	28.0	15.0
Cytochrome <i>c</i> reductase*	5.9	3.0	3.1
Succinate dehydrogenase*	73.0	49.0	13.0
Log ₁₀ scrapie titer†	9.4	9.1	8.8
Volume (ml)	58	100	45

* One unit of enzyme activity = 1 μ mol of product formed per min at the temperature indicated in the text.

† Total scrapie infectious units (ID₅₀) determined by mouse end-point quantal titration assays.

prepared with phosphate-buffered saline that contained 10% fetal calf serum, penicillin at 0.5 unit/ml, streptomycin at 0.5 μ g/ml, and amphotericin at 2.5 μ g/ml. For each dilution, six mice were inoculated intracerebrally with 0.03 ml of the diluted suspension. The animals were then examined weekly during the next 12 months for clinical signs of scrapie, i.e., bradykinesia, plasticity of the tail, and a coarse, ruffled appearance of the coat. Histologic examination of the brain and spinal cord was done only occasionally to confirm the clinical diagnosis. Titers were calculated by the method of Spearman and Kärber (22). The standard deviations of the titers varied between 0.2 and 0.5 log ID₅₀ units.

RESULTS

The distributions of subcellular markers in the scrapie-infected spleen homogenate, the 121 × g pellet, and the combined 121 × g supernatant, prepared as described above, are shown in Table 1. Approximately 54% of the total protein and 72% of the RNA were found in the 121 × g supernatant fluid, whereas only 4% of the DNA was found in this fraction. Nearly 70% of the SDH activity and 55% of the LDH activity were found in the 121 × g supernatant fluid, while 65% of the acid phosphatase and 51% of the cytochrome *c* reductase activities were found in the supernatant fraction. For alkaline phosphatase and 5'-nucleotidase, 44% and 48% of the activities were found in the 121 × g supernatant, but only negligible activities were noted in the 121 × g pellet. Thus, a significant degree of inactivation of these enzyme activities appears to have occurred during the initial preparative procedure. After centrifugation, the majority of the scrapie agent assayed by infectivity titers was found in the supernatant fraction.

The 121 × g supernatant fluid was used for the analytical differential centrifugation studies that are described below. This supernatant was divided into three aliquots: one received no further treatment, the second was sonicated for three 15-sec bursts at 4° with 1 min of cooling between each burst, and the third was treated with 0.5% DOC (wt/vol).

The sedimentation profiles shown in Fig. 1 are for SDH and LDH under the three conditions described. SDH activity was cleared from the supernatant fraction at relatively low $\omega^2 t$ values, as would be expected for a mitochondrial marker. The

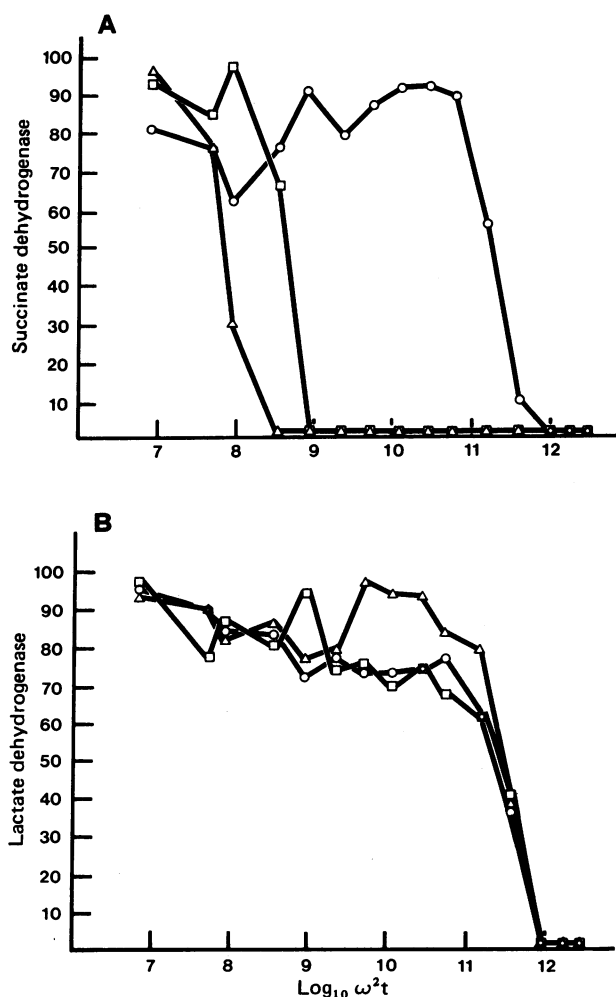


FIG. 1. Sedimentation profiles of succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) in 121 × g supernatants of scrapie-infected murine spleen. Samples were untreated (Δ), sonicated (\square), or treated with 0.5% DOC (\circ). Activities are in relative units; a relative activity of 100 for (A) SDH and (B) LDH represents 0.26 and 2.90 enzyme units, respectively. One unit of enzyme is the amount that catalyzes conversion of 1.0 μ mol of substrate to product per min under standard assay conditions.

degree of sonication used in these experiments appeared to disrupt the mitochondrial structure only slightly, so that only a small shift in SDH activity to the right was apparent (23). Virtually complete destruction of the mitochondria, however, was achieved by the addition of 0.5% DOC, which shifted the SDH activity profile from an $\omega^2 t$ value of about 10^7 to about 10^{12} rad²/sec. These data indicated that high speeds of centrifugation for long periods were required to pellet the SDH activity in the presence of DOC. In contrast, the profile of LDH activity (Fig. 1B) was unaffected by sonication or treatment with DOC, as would be expected for a "soluble" protein; sedimentation of the LDH activity required an $\omega^2 t$ value of about 10^{12} rad²/sec. All profiles for LDH were similar to the profile for SDH observed after treatment with DOC. It is noteworthy that all curves for SDH and LDH activities fell over a narrow range of $\omega^2 t$ values, which indicated that the particles containing these enzymatic activities were relatively homogeneous in size.

In Fig. 2 the sedimentation profiles for alkaline phosphatase, acid phosphatase, and glucose-6-phosphatase are shown. In each case the enzymatic profile for the untreated homogenate showed a gradual fall over the entire range of $\omega^2 t$ values from

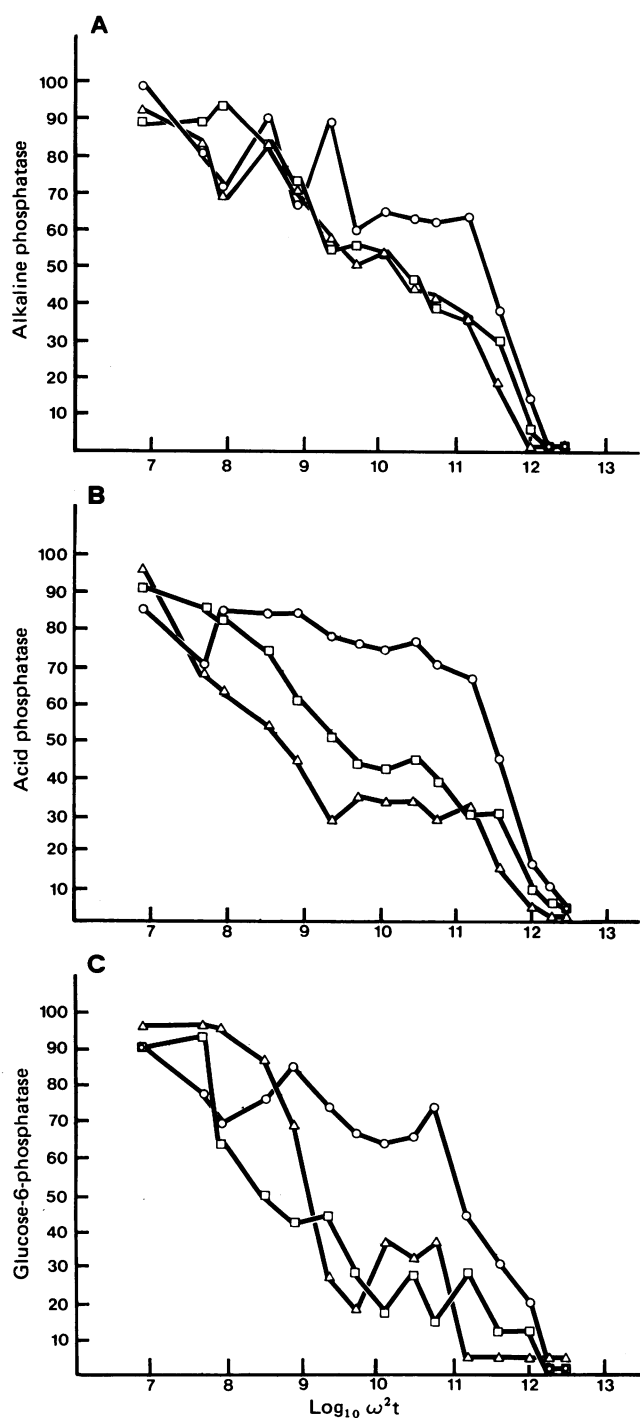


FIG. 2. Sedimentation profiles of alkaline phosphatase, acid phosphatase, and glucose-6-phosphatase activities in $121 \times g$ supernatants of scrapie-infected murine spleen. Samples were untreated (Δ), sonicated (\square), or treated with 0.5% DOC (\circ). A relative activity of 100 for (A) alkaline phosphatase, (B) acid phosphatase, and (C) glucose-6-phosphatase represents 0.033, 0.020, and 0.017 enzyme units, respectively.

10^7 to 10^{13} . Generally, sonication shifted the pattern only slightly to the right, whereas the addition of DOC removed these enzymes from their membrane-bound locations and gave a pattern very similar to that observed for LDH. The rather gradual decrease in the activities of alkaline phosphatase, acid phosphatase, and glucose-6-phosphatase over the range of $\omega^2 t$ values from 10^7 to 10^{12} indicated that these enzymatic activities were associated with particles of various sizes. Because 0.5%

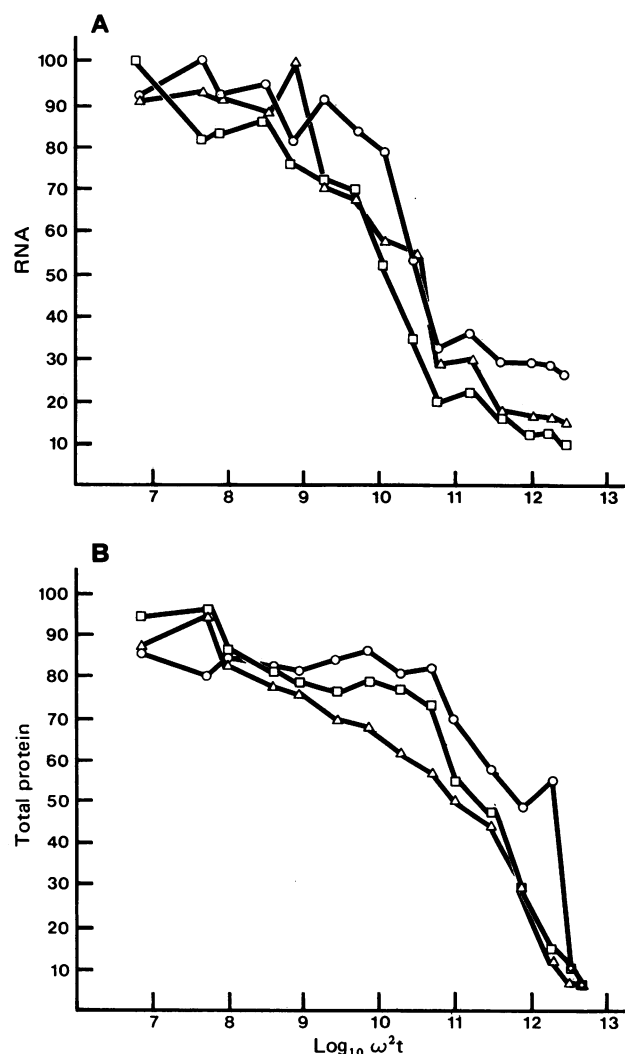


FIG. 3. Sedimentation profiles of RNA and total protein in $121 \times g$ supernatants of scrapie-infected murine spleen. Samples were untreated (Δ), sonicated (\square), or treated with 0.5% DOC (\circ). For (A) RNA and (B) total protein, a value of 100 relative units equals 0.79 mg and 9.3 mg, respectively.

DOC reduced the size of these particles to that of soluble proteins, it was reasonable to assume that the sedimentation behavior of these enzyme markers in untreated homogenates was due primarily to their attachment to cellular membranes.

Profiles for RNA and total protein sedimentation are shown in Fig. 3. In contrast to the previously discussed markers, the profile for RNA exhibited a steep fall over a range of $\omega^2 t$ values from 10^9 to 10^{11} , and the addition of DOC caused only a minor shift to the right. Because about 85% of cellular RNA is ribosomal, the RNA sedimentation profile mainly reflects the sedimentation behavior of ribosomes (22, 24, 25). The minor shift in the profile for RNA after DOC addition indicated that most of the ribosomes in the $121 \times g$ supernatant fluid were free and not bound to membranes (24–27). Profiles for total protein showed a small shift to the right upon sonication and addition of DOC, as would be expected (Fig. 3B).

Comparative studies showed that the sedimentation profiles for subcellular markers in spleen suspensions of mice infected with scrapie did not differ from the profiles of uninoculated controls.

In Fig. 4 sedimentation profiles for scrapie infectivity are depicted. As shown in Fig. 4A, the scrapie infectivity in an

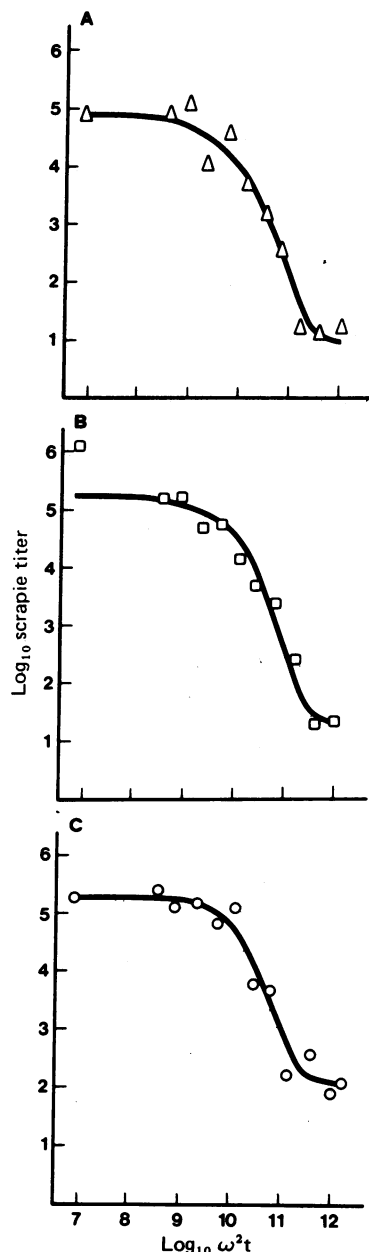


FIG. 4. Sedimentation profiles of the scrapie agent in $121 \times g$ supernatants of scrapie-infected murine spleen. Samples were (A) untreated (Δ), (B) sonicated (\square), or (C) treated with 0.5% DOC (\circ). Scrapie titers are given as ID₅₀/30 μ l of intracerebral inoculum.

untreated homogenate disappeared from the supernatant over a relatively narrow range of $\omega^2 t$ values from 10^9 to 10^{11} rad²/sec.

Fig. 4 B and C shows that the sedimentation profiles of the scrapie agent were not substantially altered by sonication or by treatment with DOC. Thus, in contrast to many of the subcellular markers, the sedimentation behavior of the scrapie agent was not greatly changed by treatment with detergent that disrupts cellular membranes. A comparison of the sedimentation profiles of the scrapie agent with those of free polyribosomes (Fig. 3 A) revealed striking similarities. Both the scrapie agent and free polyribosomes progressively sedimented at $\omega^2 t$ values between 10^9 and 10^{11} rad²/sec. In addition, neither sonication nor treatment with detergent shifted substantially the sedimentation profiles of either. In Table 2 the $\omega^2 t$ values corresponding to a 1 log decrement in infectivity titers were

Table 2. Estimated sedimentation coefficients of the scrapie agent

Preparation	$\omega^2 t$ value for 1 log infectivity decrement,* (rad ² /sec) $\times 10^{-10}$	Estimated $s_{20,w}$,† Svedberg units
Untreated	1.0	1200
Sonicated	2.0	600
DOC-treated	3.0	400

* Determined from visually fitted curves shown in Fig. 4.

† Calculated according to Anderson (8). See text.

calculated from the visually fitted curves in Fig. 4. These values represented the centrifugation speeds and times required to sediment 90% of the scrapie agent in a fixed-angle rotor and appeared to increase with sonication and detergent treatment.

Utilizing the methodology of Anderson (8), sedimentation coefficients ($s_{20,w}$) for the scrapie agent in untreated, sonicated, and DOC-treated samples were estimated to be 1200, 600, and 400, respectively. These $s_{20,w}$ calculations for the agent assumed that the agent has a density of 1.2 g/cm³ (refs 28 and 29; S. B. Prusiner, unpublished observations). Corrections for the viscosity and density of the centrifugation media considered only the 8.5% sucrose in the homogenization buffer.

DISCUSSION

In order to develop a protocol directed toward purification of the scrapie agent, the sedimentation properties of the agent have been determined in a fixed-angle rotor. The data derived from these experiments are directly applicable to development of procedures for fractionating scrapie-infected tissue. The sedimentation profiles for the scrapie agent clearly illustrate that centrifugation at $\omega^2 t$ values of less than 10^9 rad²/sec allows the agent to remain in the supernatant. At $\omega^2 t$ values of 10^{11} rad²/sec or greater, virtually all of the scrapie agent is sedimented. The sedimentation profiles of the agent do not change substantially when it is sonicated or treated with 0.5% DOC. Treatment with DOC disrupts cellular membranes in the spleen and solubilizes all of the membrane-bound markers measured. These solubilized membrane markers have a sedimentation profile much like that of LDH. Thus, the studies presented here indicate that the sedimentation behavior of the scrapie agent is largely independent of cellular membranes and is not substantially affected by detergents that destroy them. Scrapie infectivity is known to be unaltered by treatment with DOC, but is substantially reduced by treatment with sodium dodecyl sulfate, which also denatures proteins (5, 6, 29, 30).

A comparison of the sedimentation behavior of the scrapie agent with that of the subcellular markers measured indicates that the agent sediments with ribosomal RNA. The sedimentation profile of ribosomal RNA indicates that most of the RNA sediments in the form of free polyribosomes, because the average $s_{20,w}$ for the RNA marker is 360 S and treatment with detergent does not substantially shift the centrifugation profile (25). Other studies indicate that the sedimentation behavior of scrapie is independent of polyribosomes, because heating homogenates containing the agent to 80° for 30 min degrades the polyribosomes, but alters neither the infectivity nor the sedimentation of the scrapie agent (S. B. Prusiner, S. P. Cochran, W. J. Hadlow, and C. M. Eklund, unpublished observations).

The scrapie agent appears to exhibit a sedimentation coefficient between 400 and 1200 S. The smaller estimates of the $s_{20,w}$ for the agent after sonication or detergent treatment suggest that the agent in untreated preparations may bind

nonessential macromolecules, which are removable, or it may exist as an oligomer that can be disaggregated. Further experiments are needed employing rate-zonal gradient centrifugation with swinging-bucket rotors to establish more precisely the sedimentation coefficient of the agent.

The operational size of the scrapie agent appears to be larger than that of poliovirus, which has an $s_{20,w}$ of 160 S, a molecular weight of 6.8×10^6 , and a diameter of about 27 nm, as judged by electron microscopy (31). The 400 S value for the scrapie agent is consistent with available filtration data, which indicate that the agent passes through a filter containing pores 50 nm in diameter, but is retained by filters having pore sizes less than 30 nm in diameter (32–34). In addition, scrapie agent infectivity was found to appear in the void volume of an agarose column that excluded the particles of molecular weight of 5×10^7 or greater (28). Ionizing radiation target size data suggesting that the scrapie agent has a molecular weight of about 10^5 are not unreasonable if it is assumed that the core needed for infectivity is quite small relative to the overall physical size of the particle (35). It is noteworthy that the unusual spherical particles found in postsynaptic terminals of the brain tissue of mice and sheep infected with scrapie agent are about 37 nm in diameter, but have a dense core 23 nm in diameter (refs. 36–38; J. R. Baringer and S. B. Prusiner, unpublished observations).

It will be of interest to see whether fractions of brain suspensions from mice infected with scrapie will exhibit sedimentation profiles similar to those observed with spleen. The work of Hunter, Millson, Kimberlin, and coworkers suggests this will be the case, since they report that greater than 90% of scrapie infectivity is sedimented in the ultracentrifuge at $150,000 \times g$ for 90 min (an $\omega^2 t$ value of 10^{10} rad²/sec) in the presence or absence of lysolecithin, a nonionic detergent (6). Because the titer of the scrapie agent in brain is usually 10–100 times higher than that found in spleen, sedimentation profiles of the agent from brain will be of great importance (5, 9).

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